JOURNAL OF VIROLOGY, Dec. 1996, p. 9055–9059 Vol. 70, No. 12
0022-538X/96/$04.00+0

Mapping of Independent V3 Envelope Determinants of Human Immunodeficiency Virus Type 1 Macrophage Tropism and Syncytium Formation in Lymphocytes

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Received 13 March 1996/Accepted 23 August 1996

The V3 region of the human immunodeficiency virus type 1 (HIV-1) envelope protein is known to have a major influence on macrophage tropism as well as the ability to cause syncytium formation or fusion in CD4-positive lymphocyte cultures. Using infectious molecular HIV-1 clones, a series of mutant clones was created which allowed detailed mapping of V3 amino acid positions involved in these properties. In these experiments the non-syncytium-inducing phenotype in T cells did not always correlate with macrophage tropism. Macrophage tropism appeared to depend on the presence of certain combinations of amino acids at five specific positions within and just outside of the V3 loop itself, whereas syncytium formation in lymphocytes was influenced by substitution of particular residues at two to four positions within V3. In most cases, different V3 amino acid positions were found to independently influence macrophage tropism and syncytium formation in T cells and position 13 was the only V3 location which appeared to simultaneously influence both macrophage tropism and syncytium formation in lymphocytes.

Human immunodeficiency virus type 1 (HIV-1) strains vary extensively in their ability to infect, fuse, and kill different cell types in vivo and in vitro. In spite of the likely importance of these differences to the tempo and types of clinical syndromes observed in patients, little is understood about exactly which viral sequence variations are most relevant to different clinical disease parameters. Previous studies of viruses isolated from individual patients at sequential time points have indicated that macrophage-tropic/non-syncytium-inducing (NSI) viruses, which predominate early during asymptomatic infection, mutate to generate non-macrophage-tropic/syncytium-inducing (SI) viruses during the course of AIDS (2, 6, 20, 26, 29, 32, 38, 39, 43, 44). This switch in viral phenotype may have relevance to progression of clinical disease, but it is unclear whether the loss of macrophage tropism or the acquisition of the SI phenotype is the more important effect in vivo or indeed whether these phenotypic changes are the cause or the effect of alterations in clinical disease (13). Although several regions of the HIV-1 genome can influence macrophage tropism (3–5, 23, 27, 28, 31, 34), the V3 region of the HIV-1 envelope protein has a major influence on both macrophage tropism and the NSI phenotype (7, 10, 15, 22, 25, 35, 40, 47) and these properties have been closely correlated in many HIV-1 isolates (38, 39).

Previous studies have shown that V3 sequences of macrophage-tropic HIV clones are relatively homogeneous, with 85 to 100% homology, in contrast to non-macrophage-tropic clones whose V3 regions are more variable (10, 36, 50). However, the V3 sequences of macrophage-tropic clones do not have unique amino acids at any positions which allow definitive identification of macrophage-tropic HIV strains by sequencing alone (10). Preliminary mapping experiments with infectious molecular clones previously showed that two to eight V3 amino acids were involved in macrophage tropism (10, 41). In contrast, other studies showed that the NSI/SI phenotype was influenced by amino acids at only one to three V3 positions (15). The present experiments used detailed mutational analysis to identify more precisely V3 amino acids involved in macrophage tropism. The results demonstrated that macrophage tropism was associated with mutation of as few as one to two amino acids at several different positions within or adjacent to the V3 loop. Macrophage tropism did not correlate with the NSI phenotype. Many viable mutant virus clones exhibited both macrophage tropism and the SI phenotype, and some clones with the NSI phenotype were non-macrophage tropic.

Although cell tropism can be influenced by several different HIV-1 genes, the present experiments focused on detailed analysis of amino acids in and near the V3 region of envelope, and all other viral sequences were kept constant. Experiments were carried out using infectious HIV-1 molecular clones constructed as previously described (10). Mutant V3 regions were generated by annealing six overlapping synthetic oligonucleotides and ligating them into the *Mlu*I and *Nhe*I sites of the plasmid p4-14 (10), derived from the non-macrophage-tropic clone NL4-3 (1). Full-length recombinant plasmids were then transfected into clone 1022 HeLa-CD4 cells (8) or phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC), and 24 h later cells were cocultivated with fresh PHA-stimulated PBMC to produce virus stocks (10, 45). In these experiments V3 regions from three previously described isolates or clones were compared. When the V3 regions cloned from isolates 13539 and 13231 (10) were inserted into the NL4-3 background to create clones 123 and 146, they showed the SI phenotype but did not infect macrophages (Table 1). In contrast, clone 10-26, containing the V3 sequences of the macrophage-tropic clone JR-CSF (30) in the background of clone NL4-3, maintained the macrophage tropism and the NSI phenotype of JR-CSF (Table 1). The V3 region of JR-CSF differed from the V3 regions of each of the SI clones by 11 different amino acids. In order to identify the particular amino acids involved in macrophage tropism, mutant V3 regions were generated in which the amino acids differing between JR-CSF and each patient-derived SI clone were altered systematically.

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TABLE 1. Differing tropism and syncytium-inducing phenotype in T cells by HIV molecular clones expressing various V3 regions

Clone	V3 source[a]	MT2[b]	HeLa-CD4[c]	Macrophages[d]
10-26	JR-CSF	NSI	<3	11,000
123	13539	SI	1,600	<7
146	13231	SI	3,100	<7
NL4-3	NL4-3	SI	3,900	<7

[a] Source of V3 sequence (*Mlu*I-*Nhe*I) inserted into clone NL4-3 (p4-14) (10).
[b] Phenotype of MT2 cells 24 h after cocultivation of 100,000 MT2 cells with 10,000 infected PBMC (26, 29).
[c] Foci per 0.2 ml in clone 1022 CD4-positive HeLa cells (8).
[d] HIV p24 (picograms/milliliter) in supernatant of infected macrophages on days 10 to 14 as described previously (45).

Comparison of JR-CSF and patient 13539 clones revealed that two different regions of V3 could separately influence macrophage tropism (Fig. 1A). In clone 256 substitution of an isoleucine for the leucine at position 30 was sufficient to cause macrophage tropism (Fig. 1A). Conversely, loss of the isoleucine at position 30 abolished macrophage tropism in clones 230, 255, and 262. However, as seen in clone 126, isoleucine at position 30 was not absolutely necessary for macrophage tropism if a lysine at position 10 and histidine at position 13 were both present (Fig. 1A). These two different regions of the JR-CSF V3 sequence induced macrophage tropism independently and showed no additive behavior when present together in clone 10-26. Therefore, it is possible that these separate V3 positions influence macrophage tropism by different mechanisms (21).

When similar mutants were made comparing the V3 regions of JR-CSF and clone 146 from patient 13231, an asparagine at position 36 and a serine at position 38 both appeared to be necessary for macrophage tropism (Fig. 1B). Interestingly, these two positions were not within the V3 loop itself but instead were situated just downstream from the C-terminal cysteine of the loop. Comparison with the data in Fig. 1A showed that asparagine at position 36 and serine at position 38 were originally present in clone 123 from patient 13539 (Fig. 1A). Similarly, the lysine at position 10 and the isoleucine at position 30 which were both shown to be important in clone 123 were already present in clone 146 from patient 13231 (Fig. 1B). Taken together, the data in Fig. 1 showed that five different positions (positions 10, 13, 30, 36, and 38) within and adjacent to the V3 region had a critical influence on macrophage tropism. Comparison of these results to previous V3 sequence data for infectious molecular clones from both brain and blood (10, 36) indicated that these critical amino acids were the most commonly occurring residues at these positions

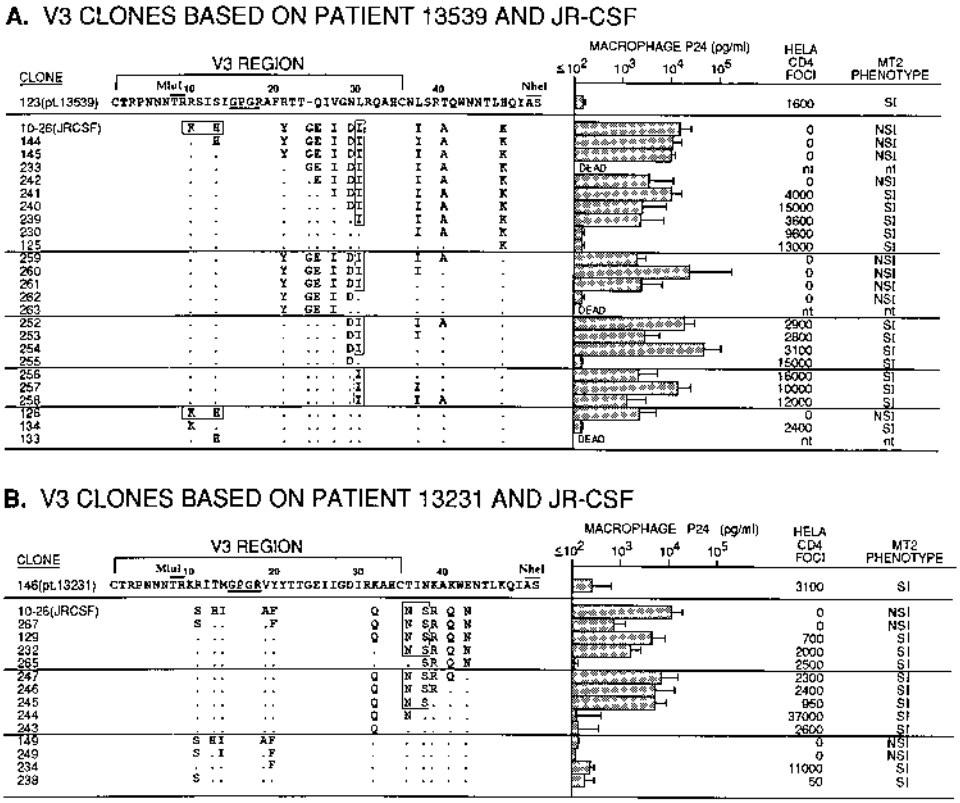

FIG. 1. Influence of V3 amino acid sequence on macrophage tropism, infectivity in HeLa-CD4 cells, and syncytium formation in MT2 leukemia cells. Infectious HIV molecular clones were derived by inserting the sequences shown between the *Mlu*I and *Nhe*I sites within or near the V3 region of the NL4-3-derived plasmid p4-14 (10). In panel A, clone 123 has the sequence from patient 13539 and in panel B, clone 146 has the sequence from patient 13231. In both panels, clone 10-26 has the sequence from JR-CSF. Amino acids critical to macrophage tropism are boxed. HIV infectivity in macrophages was assessed by p24 assay of culture supernatant on days 7, 10, 14, and 18 after infection of cultures with 1,000 50% tissue culture infectious units of HIV-infected PBMC as described previously (45). Data shown are the geometric means and standard errors of three to five separate experiments using the p24 values from day 14 postinfection. Clones were considered macrophage tropic if p24 values were greater than 1,000 pg/ml. It should be noted that non-macrophage-tropic viruses had p24 values above background, usually in the range between 100 and 300 pg/ml. Infectivity for clone 1022 HeLa-CD4 cells was analyzed by focal infectivity assay as previously described (8). Values shown are geometric means of focus-forming units per 0.2 ml. Phenotype in MT2 cells was analyzed by cocultivation of 100,000 MT2 cells with 10,000 HIV-infected PBMC in 48-well plates. Massive syncytium formation (SI phenotype) was detectable by observation of cultures with an inverted microscope after 24 to 48 h of cocultivation.

TABLE 2. Amino acid sequences of 29 patient-derived clones at five V3 positions involved in macrophage tropism

Tropism	No. of patients	V3 position[a]				
		10	13	30	36	38
Macrophage	7	K	H	I	N	S
Macrophage	3	K	S	I	N	S
Macrophage	2	R	P	I	N	S
Macrophage	2	K	P	I	N	S
Macrophage	2	R	H	I	N	S
Macrophage	2	K	N	I	N	S
Macrophage	1	R	N	I	N	S
Macrophage	1	K	G	I	N	S
Macrophage	1	G	P	I	N	S
Macrophage	1	K	H	L	N	S
Non-macrophage	1	I	H	I	N	S
Non-macrophage	1	R	S	L	N	S
Non-macrophage	1	K	R	M	N	S
Non-macrophage	1	R	H	I	T	N
Non-macrophage	1	R	H	M	T	N
Non-macrophage	1	K	T	I	T	N
Non-macrophage	1	R	P	L	S	S

[a] Boxed sequences are from three patient clones, studied by mutational analysis to demonstrate importance of residues 10, 13, 30, 36, and 38 in macrophage tropism (Fig. 1). Complete V3 sequences and their effects on macrophage tropism were published previously (10, 36, 37).

in macrophage-tropic clones (Table 2). Macrophage-tropic clones from 7 of 22 patients matched the amino acid sequence associated with macrophage tropism at all five of the critical positions identified so far. All macrophage-tropic clones from these patients had asparagine at position 36 and serine at position 38 just outside V3, and all but one patient had isoleucine at position 30. This latter patient had leucine at position 30 plus lysine at position 10 and histidine at position 13, and thus matched mutant clone 126 (Fig. 1A) at all five positions. In contrast, the non-macrophage-tropic clones were different in each patient studied, and none matched the macrophage-tropic sequence at all five positions. One patient in this group did match at four of the five positions, but this patient had isoleucine at position 10 which was not seen in any other individual (Table 2). These comparisons demonstrated clearly that the V3 residues identified as important for macrophage tropism in Fig. 1 correlated well with macrophage tropism in patient virus isolates. However, these results do not allow a precise definition of all the V3 sequence motifs associated with macrophage tropism. Additional mutants would have to be constructed to compare the macrophage-tropic consensus V3 sequence with more non-macrophage-tropic patient sequences in order to explore the effects of different conformations on this phenotype.

Interactions between V3 and V1/V2 regions of envelope protein are known to influence specificity of neutralizing antibodies and biological phenotype. In order to examine whether such interactions might influence the phenotypes of our V3 mutant clones we have inserted a different V1/V2 region (45) from the HIV-1 clone, Ba-L, into two of the macrophage-tropic SI clones (232 and 247) described in Fig. 1. The results indicate that the original phenotype of these clones was not altered by these different V1 and V2 sequences (data not shown). Of course, it should not be forgotten that envelope sequences other than V1, V2, and V3 and also other HIV genes can influence macrophage tropism (3–5, 27, 31, 34). Although keeping the background genes constant was obviously required in the present experiments in order to study the influence of complex V3 variations, it is possible that the background genes in the clones used could still influence these results. Thus, it may be interesting to examine these V3 sequences in other genomic backgrounds to study additional possible interactions between V3 and non-V3 sequences.

The same mutants described above were also analyzed for infectivity in HeLa-CD4 cells (8) and SI/NSI phenotype in the MT2 T-cell leukemia cell line (21, 24). In all cases the SI phenotype in MT2 cells correlated with positive infectivity in HeLa-CD4 cells. However, in contrast to what was seen with the parental clones (Table 1), there was no correlation between macrophage tropism and the NSI phenotype. For example, in Fig. 1A nine clones had tropism for both macrophages and HeLa-CD4 cells and had the SI phenotype in MT2 cells, and one clone, 262, was negative for infectivity in both macrophages and HeLa-CD4 cells and had the NSI phenotype in MT2 cells. Similar results were seen with the clones in Fig. 1B. Based on data in Fig. 1A, the SI phenotype in patient 13539 required both a serine at position 13 and a glutamine at position 25 (Fig. 2). Similarly, the SI phenotype in patient 13231 required either an arginine at position 11 or a tyrosine at position 20 plus a threonine at position 13 and a methionine at position 14 (Fig. 2). The requirement for both threonine and methionine in these combinations was not proven since clones containing all possible combinations at these four positions were not tested.

The present results of mapping the SI phenotype in these two patients are in agreement with previous data from others who identified similar amino acids as being important for the SI phenotype (15, 22). However, the influence on SI phenotype of the substitution of the slightly negatively charged tyrosine for the neutral phenylalanine at position 20 (compare clones 249 and 238) has not been described previously. It should be noted that the substitution of tyrosine at position 20 was not required for the SI phenotype (clone 234 in Fig. 1B). However, this substitution was of particular interest because in earlier cases analyzed the SI phenotype was usually associated with the presence of a more positively charged residue (15, 22). These independent constraints for specific amino acids at each V3 position influencing SI phenotype, rather than a general requirement for more positively charged residues, suggest that protein conformation of the V3 region may be important in determining the SI phenotype. This conclusion is also supported by the recent report that positively charged amino acids were not found at these critical V3 positions in SI viruses of HIV subgroup F (24).

The most striking finding in the present experiments was that in most cases the V3 amino acid positions influencing macrophage tropism did not coincide with those influencing the SI/NSI phenotype in T cells and HeLa-CD4 cells (Fig. 3). Thus, there was no precise correlation between macrophage tropism and the NSI phenotype in T cells. Only position 13 in patient 13539 appeared to influence both macrophage tropism

		V3 AMINO ACID					PHENOTYPE		
PATIENT	CLONE	11	13	14	20	25	HeLa-CD4	MT2	MACROPHAGE
JR	JRCSF	S	H	I	F	E	0	NSI	+
13539	242	S	(S)	I	F	E	0	NSI	+
	241	S	(S)	I	F	(Q)	4000	SI	+
	126	S	H	I	F	(Q)	0	NSI	+
13231	249	S	(T)	I	F	E	0	NSI	−
	234	(R)	(T)	(M)	F	E	11,000	SI	−
	238	S	(T)	(M)	(Y)	E	50	SI	−

FIG. 2. Influence of V3 amino acids 11, 13, 14, 20, and 25 on SI phenotype and HeLa CD4 tropism. Circled amino acids differ from clone JR-CSF.

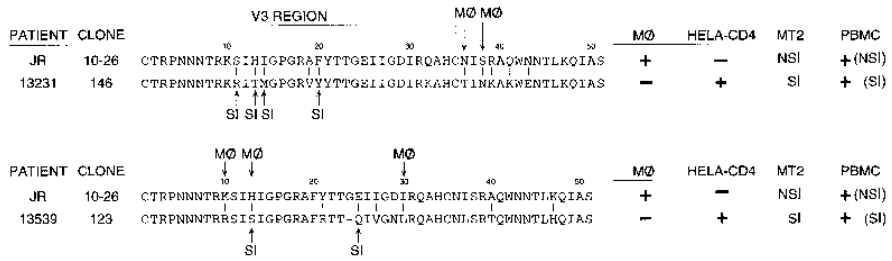

FIG. 3. Summary of HIV envelope amino acid positions within and near the V3 region which influence macrophage tropism (Mϕ) and NSI/SI phenotype in T cells.

and the NSI phenotype. These results suggest that V3 acts by different mechanisms on macrophage tropism and the NSI phenotype, but at present it is unclear what these mechanisms are. Macrophage tropism often acts at the level of HIV entry into cells and may work by interactions of specific V3 sequences with a macrophage-specific protease or coreceptor. In contrast, NSI and SI are fusion phenotypes and although fusion often correlates with the ability to enter a particular cell type, NSI strains of HIV can enter and infect PBMC even though they do not cause massive cell fusion. This could be explained by a quantitative difference in the extent of fusion induced by any particular V3 sequence, but a biochemical basis for this has not yet been determined. The present results mapping these phenotypes to separate V3 amino acids should facilitate a more focused biochemical analysis of these different mechanisms.

Recently, experiments by several groups have identified four different coreceptors, fusin, CKR2b, CKR3, and CKR5, which together with CD4 are important for entry of HIV-1 into target cells (11, 16–19). T cell-tropic/SI virus strains appear to use fusin, whereas macrophage-tropic/NSI virus strains use CKR2b, CKR3, or CKR5. One dual-tropic (macrophage-tropic/SI) virus strain was able to use either fusin or CKR5 as a coreceptor (17). Sequence differences in the V3 region of HIV-1 envelope determine in part the specificity of interactions with each of these coreceptors (11). The present data shows that macrophage tropism and the NSI phenotype are distinct phenotypes influenced by different V3 amino acid sequences. This raises the question of which set of V3 sequences is actually involved in the interaction with the coreceptors used by macrophage-tropic/NSI viruses found frequently in primary patient isolates. Furthermore, these data suggest the possibility that non-macrophage-tropic/NSI viruses such as clones 149, 249, and 262 (Fig. 1), which have not been recognized previously, may require a different coreceptor from those defined so far. Future experiments using the mutant V3 clones shown in Fig. 1 should allow a precise definition of which V3 amino acids are critical for interactions with each of these coreceptors.

Analysis of mutant clones in the present study was able to distinguish four different viral phenotypes based on cell tropism and fusion properties. Previous data suggested that most patients are initially infected by macrophage-tropic viruses with the NSI phenotype (10, 33, 42, 46, 48–50). During disease progression, non-macrophage-tropic viruses with the SI phenotype are generated by mutation and these appear to be selectively expanded during the later stages of AIDS. The main event favoring generation of mutants would appear to be killing of infected cells by virus which would select for viruses which continue to spread to new cells and generate mutants during repeated cycles of reverse transcription (13). Although the factors favoring selection of SI viruses during the AIDS disease course are not understood, one might expect that during the transition from macrophage-tropic/NSI viruses to non-macrophage-tropic/SI viruses that macrophage-tropic/SI viruses and non-macrophage-tropic/NSI viruses might be intermediate forms. Although similar mixed phenotypes have been previously detected in HIV isolates from patients, the heterogeneity of viruses in such isolates prevents conclusive interpretation of these data (38, 39). In fact, in the one patient-derived HIV-1 molecular clone with such a mixed phenotype the macrophage tropism was determined by a V3-independent region (14, 27). It is possible that such strains exist in the heterogeneous virus populations isolated from patients, but they may be selectively lost during in vitro passage and biological cloning. Alternatively, such strains might have a selective disadvantage in vivo and thus be difficult to isolate. The current identification of V3 amino acids involved in macrophage tropism and fusion of T cells should now facilitate distinguishing these parameters and allow correlations to be made with clinical disease state or prognosis.

Previous studies with murine retroviruses have shown that pseudotyping between two murine retroviral strains with differing cell tropism appears to be important in acceleration of the leukemogenic process (9, 12). Such pseudotyping can act by causing a switch in cell tropism or by overcoming interference due to previous infection with another retrovirus. The four phenotypes of HIV described in the present report might undergo similar pseudotyping to direct HIV genomes to different cell types and thus alter pathogenicity. For example, non-macrophage-tropic/NSI strains pseudotyped into macrophages might be more easily able to maintain a latent status. Conversely, macrophage-tropic/SI strains might have increased pathogenic potential in either macrophages or T cells on the basis of fusion properties in vivo.

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